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Phosphopeptide Enrichment with TiO₂-Modified Membranes and Investigation of Tau Protein Phosphorylation

Yu-Jing Tan¹, Dexin Sui², Wei-Han Wang¹, Min-Hao Kuo², Gavin E. Reid^{1,2}, and Merlin L. Bruening^{1,*}

¹Department of Chemistry, Michigan State University, East Lansing, Michigan 48824

²Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan 48824

Abstract

Selective enrichment of phosphopeptides prior to their analysis by mass spectrometry (MS) is vital for identifying protein phosphorylation sites involved in cellular regulation. This study describes modification of porous nylon substrates with TiO₂ nanoparticles to create membranes that rapidly enrich phosphopeptides. Membranes with a 22-mm diameter bind 540 nmol of phosphoangiotensin and recover 70% of the phosphopeptides in mixtures with a 15-fold excess of non-phosphorylated proteins. Recovery is 90% for a pure phosphopeptide. Insertion of small membrane disks into HPLC fittings allows rapid enrichment of 5 mL of 1 fmol/μL phosphoprotein digests and concentration into small-volume (10's of μL) eluates. The combination of membrane enrichment with tandem mass spectrometry reveals seven phosphorylation sites from *in vivo* phosphorylated tau (p-tau) protein, which is associated with Alzheimer's disease.

Phosphorylation of serine, threonine, or tyrosine residues is one of the most important mechanisms for creating protein diversity in eukaryotic cells.¹ Remarkably, about one-third of human proteins are substrates for phosphorylation at some stage in their life cycle,² and reversible phosphorylation plays a key role in regulating cell processes such as signal transduction and metabolism.^{3,4} Thus, a fundamental understanding of regulatory pathways requires identification of protein phosphorylation states. Currently, the leading technology for evaluating protein phosphorylation is MS,⁵ due to its high speed and sensitivity, and the ability in some cases to determine the location of the phosphorylation site by tandem mass spectrometry (MS/MS) methods.⁶ However, although reversible phosphorylation is ubiquitous in cells, phosphorylation is intrinsically a dynamic and transient property,⁷ so phosphoproteins are generally present at relatively low abundances and low stoichiometries compared to non-phosphoproteins.⁸ To partially overcome the low abundance, a number of groups developed methods to enrich phosphopeptides from digested protein mixtures.^{9–14} Most current phosphoprotein characterization occurs by electrospray ionization (ESI) or matrix assisted laser desorption/ionization (MALDI) MS following an initial phosphoprotein or phosphopeptide enrichment procedure.^{15–17}

Phosphopeptide enrichment methods include immunoprecipitation, covalent immobilization, and adsorption to metal oxide resins or immobilized metal-ion complexes.^{7,18–20} All of

*To whom correspondence should be addressed. bruening@chemistry.msu.edu; phone: (517) 355-9715 x 237.

SUPPORTING INFORMATION AVAILABLE

Details on chemicals, membrane modification, digestion, enrichment, and mass spectrometry procedures, analysis of TiO₂ immobilization, mass spectra of digested proteins, and tables of peptides identified from mass spectra. This information is available free of charge via the Internet at <http://pubs.acs.org>.

these methods are useful, and they often provide complementary subsets of phosphopeptides.^{21,22} The most common phosphopeptide enrichment strategy is affinity chromatography. First introduced by Andersson and Porath in 1986,²³ immobilized metal affinity chromatography using Fe^{3+} complexes (IMAC-Fe) has been the most common method to enrich phosphopeptides. IMAC-Fe relies on hard acid-hard base interactions between positively charged metal-ligand complexes and specific negative functional groups such as phosphates. Other candidate metal ions include Al^{3+} , Ga^{3+} , Co^{3+} and Ti^{4+} ,¹⁸ and this technique is widely implemented with silica columns,²⁴ magnetic beads²⁵ and modified MALDI plates.²⁶ More recently, many studies show that phosphopeptide adsorption to metal oxide resins is particularly attractive for rapid and highly specific enrichment if binding conditions are carefully controlled.^{17,27} At low pH, TiO_2 , the metal oxide most commonly used for phosphopeptide enrichment, has a positively charged surface that selectively adsorbs phosphorylated species, and the adsorbed species subsequently elute under basic conditions.²⁸ Addition of excluders and low-pH buffer to the phosphopeptide solution helps to alleviate nonspecific binding of acidic peptides to TiO_2 .^{29,30}

This paper describes the functionalization of porous nylon substrates with a thin layer of TiO_2 nanoparticles to create membranes for phosphopeptide enrichment.³¹ Adsorption of phosphopeptides from solutions passing through the membrane (Figure 1) followed by rinsing and elution yields concentrated solutions of isolated phosphopeptides. The sub-micron pore size in the nylon allows phosphopeptide adsorption without mass transfer limitations, and the high surface areas of both the nylon substrate and the nanoparticles give materials with a high phosphopeptide binding capacity.³⁰ Moreover, because of the minimal membrane thickness, the modified membranes rapidly capture phosphopeptides from relatively large volumes (1 mL or 5 mL) of dilute (5 fmol or 1 fmol phosphopeptide/ μL) solutions with minimal backpressure. Elution volumes of 10 μL lead to 100-fold increases in phosphopeptide concentration after enrichment.

In contrast to global phospho-proteomics strategies that use strong cation-exchange fractionation, metal oxide affinity chromatography, and liquid chromatography (LC)-MS,^{17,32} our interests focus on investigating phosphorylation in a few proteins associated with a specific pathway or pathology, for instance the role of tau protein phosphorylation in Alzheimer's disease.^{33,34} To demonstrate the utility of TiO_2 -modified membranes, we examine phosphopeptide enrichment from a digest of tau protein that was phosphorylated by glycogen synthase kinase-3 β (GSK-3 β). The combination of membrane enrichment and tandem MS reveals seven phosphorylation sites from in vivo phosphorylated tau protein, three of which seem dominant. Collisional Induced Dissociation (CID), Higher-Energy Collisional Dissociation (HCD) and Electron Transfer Dissociation (ETD) tandem MS comprehensively confirm the results.

EXPERIMENTAL SECTION

Materials and Solutions

The supporting information gives a complete list of the reagents employed in this study. Human angiotensin II (DRVYIHPF) and phosphorylated angiotensin II (DRVpYIHPF) were acquired from Calbiochem. $\text{CH}_3\text{CH}_2\text{CO-LFTGHPEpSLEK}$ (H_5 peptide) and $\text{CD}_3\text{CD}_2\text{CO-LFTGHPEpSLEK}$ (D_5 peptide) were prepared using manual stepwise Fmoc-based solid-phase peptide synthesis as described previously.²⁶ Enrichment employed loading buffers containing 0.1 vol % trifluoroacetic acid (TFA), 49.9 vol % deionized water and 50 vol % acetonitrile (loading buffer I) or 2 vol % TFA, 48 vol % deionized water and 50 vol % acetonitrile (loading buffer II). Washing buffer I and II have the same compositions as loading buffers I and II, respectively. The elution buffer contains 0.5 vol % ammonium

hydroxide (NH_4OH), 49.5 vol % deionized water and 50 vol % acetonitrile, and has a pH of 10.9.

Membranes and Holders

Membrane modification and binding capacity studies employed an Amicon cell (model 8010, Millipore) that exposes a membrane area of 3.8 cm^2 . A peristaltic pump pulled solutions through the membrane. The unmodified nylon membranes (25 mm-diameter, nominal pore size of $0.45 \mu\text{m}$) were obtained from Millipore (HNWP02500). For experiments with small sample volumes, a 6 mm-diameter piece of membrane was placed in a flangeless fitting system (A-424, Upchurch Scientific) connected to 1/16 inch OD tubing via ferrules. The membrane was supported by a frit in the holder. The membrane area exposed to sample (0.02 cm^2) was determined by passing a dye solution through the apparatus and measuring the diameter of the colored membrane area with calipers. Sample injection occurred via a syringe pump using a 1-mL or 100- μL syringe (Figure 1).

Synthesis of Titania Nanoparticles

TiO_2 nanoparticles were prepared by the controlled hydrolysis of titanium isopropoxide.³⁵ $\text{Ti}(\text{OCH}(\text{CH}_3)_2)_4$ (1.25 mL) was dissolved in 25 mL of absolute ethanol, and this solution was added dropwise under vigorous stirring to 250 mL of water previously adjusted to pH 1.5 with nitric acid. The resulting transparent colloidal suspension was stirred overnight, and then stored in a refrigerator prior to use.

Membrane Modification

Modification of UV/ozone-cleaned (10 min) nylon membranes with titania nanoparticles occurred through consecutive adsorption of poly(sodium 4-styrene-sulfonate) (PSS) and Titania. Ten mL of a 0.02 M (with respect to the repeating unit) PSS, 0.5 M NaCl solution was first passed through the membrane at 2 mL/min, followed by 30 mL of deionized water at the same flow rate. Subsequently, a 10 mL aliquot of the titania suspension was circulated through the membrane at 1 mL/min for 30 min. Prior to passing through the membrane, the titania suspension was centrifuged for 5 min at 1300 g to remove large particles. After TiO_2 deposition, the membrane was rinsed with 30 mL of deionized water at 2 mL/min. A second PSS/ TiO_2 bilayer was deposited similarly.

Collection of Breakthrough Curves and Determination of Binding Capacity

In the breakthrough experiments, 50 mL of 20 μM phosphoangiotensin was passed through a nylon membrane modified with a $(\text{PSS}/\text{TiO}_2)_2$ film, and the effluent was collected in 2.5 mL aliquots. The flow rate was 2 mL/min. Phosphoangiotensin concentrations in each aliquot were determined by MALDI-MS using angiotensin as an internal standard. The amount of bound phosphoangiotensin was determined from the difference in feed and effluent concentrations in each aliquot.

Membrane Reusability and Single Phosphopeptide Recovery

Five mL of 4 μM phosphoangiotensin in loading buffer I was passed through a large membrane at 2 mL/min followed by 5 mL of washing buffer I and 5 mL of elution buffer at the same flow rate. Finally the membrane was reconditioned with 10 mL loading buffer I, and the same experiment was repeated nine additional times. The loading solution and eluate were analyzed by MALDI-MS to determine recovery.

Determination of Phosphopeptide Recovery Using Labeled Peptides

Ten pmol of H_5 peptide ($\text{CH}_3\text{CH}_2\text{CO-LFTGHPEpSLEK}$) and 150 pmol of BSA digest were dissolved in 100 μL of loading buffer I and passed through the small (0.02 cm^2) membrane

at 10 $\mu\text{L}/\text{min}$. The membrane was washed with 500 μL of washing buffer I at 50 $\mu\text{L}/\text{min}$, and peptides were eluted with 30 μL of elution buffer at 3 $\mu\text{L}/\text{min}$. One μL of eluate (333 fmol of H₅ peptide assuming 100% recovery) and 1 μL of internal standard (500 fmol of D₅ peptide/ μL) were spotted on a MALDI plate followed by 1 μL of matrix solution (40 mg/mL of 2,5 DHB in 1% TFA, 49% H₂O, 50% ACN). The ratio of MALDI-MS signals from the H₅ and D₅ peptides was compared to a calibration curve (Figure S1, figure numbers beginning with S refer to the supporting information) to determine the concentration of the H₅ peptide in the eluate.

Phosphopeptide Enrichment from Protein Digests

The supporting information describes the protein digestion procedure in detail. For analysis of phosphopeptides in a large excess of non-phosphopeptide, 15 pmol of α -casein, β -casein, and ovalbumin digests and 1500 pmol of BSA digest were dissolved in 500 μL loading buffer II. This solution was passed through the small-scale TiO₂-modified nylon membrane at 50 $\mu\text{L}/\text{min}$, and the membrane was washed with 3 mL of washing buffer II at 100 $\mu\text{L}/\text{min}$. Finally the phosphopeptides were eluted from the membrane with 10 μL of elution buffer at 2 $\mu\text{L}/\text{min}$.

Enrichment of Dilute Phosphopeptides with Membranes and Titansphere™ Spin-Tips

One mL of loading buffer I containing 5 fmol/ μL of digested α -casein, β -casein, and ovalbumin (or 5 mL of 1 fmol/ μL digests) was passed through the small-scale TiO₂-modified nylon membrane at 0.1 mL/min. The membrane was washed with 100 μL of washing buffer I at 10 $\mu\text{L}/\text{min}$, and the phosphopeptides were eluted with 10 μL of elution buffer at 3.33 $\mu\text{L}/\text{min}$. For comparison, similar enrichment was performed using a Titansphere™ Phos-TiO Kit following the manufacturer's instructions (see the Supporting Information for details) with two noted modifications. One mL of a solution containing 5 fmol/ μL of digested α -casein, β -casein, and ovalbumin in 0.3 vol % TFA, 25 vol % lactic acid, and 60 vol % ACN was spun through the column in five 200- μL aliquots. (Instead of using 1,000 x g to spin down a 100 μL solution in 10 min, we used 2,000 x g to spin down 200 μL of solution in 10 min.) After multiple washing steps, instead of using 50 μL of 5 % NH₄OH and 50 μL of 5 % pyrrolidine as the elution buffer, we only used 10 μL of 5 % NH₄OH to elute phosphopeptides for 5 min. The pyrrolidine is incompatible with MALDI-MS, and elution appeared complete with just NH₄OH.

Enrichment of phosphopeptides from digested p-tau

The supporting information describes the purification of p-tau, and Figure S2 shows the SDS-PAGE separations of tau and p-tau. After in-solution digestion of 100 μg of purified protein, 160 pmol of p-tau digest was dissolved in 200 μL of loading buffer II and passed through a small-scale TiO₂-modified membrane at 10 $\mu\text{L}/\text{min}$. The membrane was washed with 200 μL of washing buffer II at the same flow rate, and phosphopeptides were eluted with 50 μL of elution buffer at 5 $\mu\text{L}/\text{min}$.

Mass Spectrometry and Data Analysis

An LTQ XL ion trap mass spectrometer equipped with a vMALDI source (Thermo Fisher Scientific, San Jose, CA) was used to perform MALDI-MS and CID-MS/MS analyses. Typically, 1 μL of sample solution was spotted onto the stainless steel MALDI plate, followed by addition of 1 μL of matrix solution (40 mg/mL of 2,5 DHB in 1% TFA, 49% H₂O, 50% ACN) and drying.

For ESI-MS analyses, 40 μL samples in 1% acetic acid, 49% H₂O and 50% methanol were loaded into a Whatman multichem 96-well plate (Sigma Aldrich) and sealed with Teflon

Ultra Thin Sealing Tape (Analytical Sales and Services, Prompton Plains, NJ). Then, using an Advion Triversa Nanomate nano-electrospray ionization (nESI) source (Advion Ithaca, NY) with a spray voltage of 1.4 kV and a gas pressure of 1.0 psi, samples were introduced into a high resolution accurate mass LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with a dual pressure ion trap, HCD cell and ETD. High resolution mass spectra, as well as all ion trap CID-, HCD- and ETD-MS/MS and -MSⁿ spectra, were acquired in positive ionization mode using the FT analyzer operating at 100,000 resolving power. All mass spectra show maximum absolute intensities in the upper right corner. Assignment of peptides resulting from protein digestion was performed manually by mass fingerprinting or by MS/MS. Phosphorylation sites were manually assigned by matching MS/MS product ions with *in silico* peptide fragments generated using ProteinProspector (<http://prospector.ucsf.edu>). See the supporting information for more details on MS and MS fingerprinting.

RESULTS AND DISCUSSION

Characterization of TiO₂-Modified Nylon Membranes

Nanoparticles are attractive adsorbents for phosphopeptides because of their high surface area to volume ratio.^{36–38} However, packing nanoparticles into columns is difficult at best, and the low particle diameters will lead to extremely high backpressures.³⁹ Immobilization of nanoparticles in membrane pores provides a simple, alternative method for exploiting the high surface area and unique adsorption properties of these materials.

TEM images show that the TiO₂ particles in this study have an average diameter of 5 nm (see Figure S3), but these particles aggregate into clusters with a diameter of 50 nm. The nanoparticle synthesis gives an anatase structure,³⁵ and Eriksson and coworkers report that anatase TiO₂ nanoparticles deposited on glass effectively enrich phosphopeptides.⁴⁰ Presumably, PSS binds to the nylon membrane through hydrophobic interactions and then adsorbs TiO₂ nanoparticles electrostatically.^{31,38,41} Previously we employed a similar method to immobilize trypsin in PSS-modified nylon membranes.⁴² Unfortunately SEM images of membrane cross-sections do not show the TiO₂ nanoparticles (Figure 2c), perhaps because of the rough texture of the nylon membrane and the small and polydisperse nanoparticle size. However, the Ti energy-dispersive X-ray spectroscopy (EDX) map in Figure 2d reveals uniform coverage of Ti over the whole cross-section of the modified membrane. Notably, the layer-by-layer method allows modification of nylon membranes with a PSS/TiO₂ film in just 30 min. Moreover, stacking of membranes in the Amicon cell permits simultaneous modification of several membranes, and a single 25-mm membrane provides 8 membranes for small-scale analysis. Most of the sulfonate groups will bind with TiO₂ to minimize the number of free cation-exchange sites in the membrane and reduce adsorption of cationic peptides. The positive zeta potential of polycation-terminated films will also decrease adsorption of such peptides.⁴³

Nitrogen adsorption isotherms show that a porous nylon membrane (diameter of 25 mm and thickness of 170 μ m) has a surface area of 3300 cm², which should facilitate the adsorption of large amounts of TiO₂ nanoparticles. Analysis of TiO₂ nanoparticle-containing solutions before and after passing them through the membrane shows that 30 μ mol of Ti (presumably as TiO₂) adsorb to each membrane (exposed membrane diameter of 22 mm) during deposition of a PSS/TiO₂ bilayer (Figure S4). Adsorption of a second PSS/TiO₂ bilayer approximately doubles the amount of Ti (55 μ mol) in the membrane, which should enhance phosphopeptide binding. For 50 nm TiO₂ particles, 55 μ mol of Ti corresponds to 7×10^8 particles per cm² of internal membrane area, assuming a TiO₂ density of 4.2 g/cm³.

Phosphopeptide Binding Capacity

Binding capacity is important in selectively capturing phosphopeptides because insufficient capacity may cause low sensitivity or selective detection of only strongly binding phosphopeptides, whereas excess capacity may result in nonspecific binding. We employed classical breakthrough experiments to determine the amount of phosphopeptide, in this case phosphoangiotensin, that a (PSS/TiO₂)₂-modified membrane can capture. As Figure 3 shows, initially the membrane captures all the phosphopeptide in the loading solution, but as the membrane becomes saturated, phosphopeptides break through and eventually no phosphopeptides bind. Integrating the difference between the feed and permeate phosphopeptide concentrations in Figure 3 yields a binding capacity of 540 nmol of phosphoangiotensin/membrane (22 mm-diameter). Based on the 55 μ mol of Ti in the membrane, the binding capacity is 126 μ mol phosphopeptides/gram of TiO₂. This result agrees reasonably well with a previous study that reported a binding capacity of 520 μ mol phenyl phosphate/gram using 40 nm TiO₂.³⁷ Phenyl phosphate is much smaller than phosphoangiotensin and may give higher binding capacities.

In most analytical applications, membrane loading is much less than saturation to avoid loss of desired analytes to the permeate. Defining dynamic capacity as the amount of binding that occurs before the permeate concentration is 10 % of that in the feed, the dynamic phosphoangiotensin-binding capacity is 365 nmol phosphopeptides per membrane. Moreover, the dynamic capacity is the same at flow rates of 2 and 4 mL/min, suggesting kinetics and diffusion do not limit binding prior to approaching saturation.

Membrane Stability and Phosphopeptide Recovery

In an initial experiment to examine membrane stability and phosphopeptide recovery, we loaded, washed and eluted phosphoangiotensin 10 times with the same membrane. Phosphoangiotensin recovery (amount in eluate/amount in loading solution) was around 90 % for all of the 10 replicates with the membrane (Figure S5). Each enrichment cycle included passing 15 mL of loading buffer, 5 mL of washing buffer and 5 mL of eluent through the membrane, and the constancy of the phosphoangiotensin recovery over ten replicates suggests that the immobilized TiO₂ is stable in the membrane.

However, recoveries from mixtures of digested proteins are frequently less than those in single-peptide solutions because nonspecific binding may block adsorption sites. To quantitatively examine recoveries in a more complex mixture, we employed H₅ (CH₃CH₂CO-LFTGHPEpSLEK) and D₅ (CD₃CD₂CO-LFTGHPEpSLEK) peptides, where the D₅ peptide spotted on the MALDI plate with the sample served as an internal standard (see Figure S1 for the calibration curve). We added 10 pmol of the target H₅ peptide (CH₃CH₂CO-LFTGHPEpSLEK) to a 150 pmol BSA digest in 100 μ L of loading buffer I, passed this mixture through a nylon membrane modified with (PSS/TiO₂)₂ (Figure S6 shows mass spectra of the solution before and after passing through the membrane), rinsed the membrane to remove non-specifically bound peptides, and eluted the phosphopeptides with 30 μ L of elution buffer. (We used a relatively large amount of H₅ peptide because only 1 out of 30 μ L of eluate is spotted on the MALDI plate.) Comparison of the H₅/D₅ peptide signal intensity ratios for reference and eluate solutions (Figure S7) indicates an overall H₅ peptide recovery >70% in the eluate. The high recovery occurs despite a 15-fold excess of BSA.

Analysis of α -Casein, β -Casein, and Ovalbumin Digests

A mixture of three phosphoproteins, α -casein, β -casein and ovalbumin, served as an initial test solution for phosphopeptide enrichment from tryptic digests, and we successfully enriched 15 phosphopeptides (Figure S8). (The large number of peptides results from some missed and nonspecific cleavages and variable alkylation.) To mimic a modestly complex

biological sample, we examined enrichment from 500 μL of loading buffer I containing 15 pmol (each) of digested α -casein, β -casein, and ovalbumin along with 1500 pmol of digested BSA. (We used a relatively large amount of digest because only 10 % of the eluate is spotted on the MALDI plate.)

To greatly reduce non-specific binding in complex mixtures, Jensen and Larsen recommend the use of 2 to 5 % TFA in loading solutions.⁴⁴ Zhou et al also suggest that 6 % TFA in the loading buffer increases the efficiency of Ti(IV)-based IMAC enrichment for phosphoprotein profiling.³² As Figure 4 shows, for our modestly complex samples 2 % TFA in 1:1 water:ACN (loading buffer II) is sufficient to nearly eliminate binding of nonphosphopeptides. Prior to enrichment, the MALDI mass spectrum of the loading solution II (1 μL of solution, 30 fmol of each phosphopeptide and 3 pmol of digested BSA) reveals only one small phosphopeptide signal, along with many signals from BSA peptides (Figure 4a). In contrast the mass spectrum of the enriched sample (Figure 4b) contains signals from 19 phosphopeptides and no non-phosphopeptide signals with $S/N > 3$. The S/N for the phosphopeptides ranges from 3 to 50. Increases in the phosphopeptide S/N stem from a 50-fold preconcentration (assuming full recovery) and a decrease in ion suppression and urea interference. The S/N of the enriched sample is about half of that for enriched digests that do not contain BSA, presumably because the higher TFA concentration decreases the efficiency of phosphopeptide binding to the TiO_2 . The peaks below m/z 1500 in Figure 4b are likely due to matrix adducts or polymer that leaches from the membrane, as the MS/MS spectra show non-peptide fragmentation patterns. (Table S1 contains m/z values, S/N , and sequences for the peptides identified from Figure 4.)

Enrichment of Low-concentration Phosphopeptides with Membranes and Titansphere™ Spin Tips

Low concentrations of phosphoproteins present a major challenge for analysis. Preconcentration of all proteins using lyophilization or spin columns may lead to very complex mixtures, and phosphoproteins in dilute solutions may adsorb to container walls. Direct phosphopeptide enrichment from relatively large volumes could reduce sample loss during handling and increase throughput. Membranes are particularly attractive for such applications because they allow rapid enrichment.

We compared enrichment of dilute phosphopeptides using membranes and Titansphere™ Phos-TiO spin tips (GL Sciences Inc).^{45,46} Although other commercial systems are available for offline phosphopeptide enrichment,^{47,48} we think the Titansphere™ Phos-TiO spin tips (200 μL) provide the best comparison for treating dilute mixtures with volumes around 1 mL. Figure 5(a and b) shows the MALDI mass spectra of peptides enriched from 1-mL solutions containing 5 fmol/ μL of digested α -casein, β -casein, and ovalbumin. The eluates from the Titansphere™ spin-tips (spectrum a) and the TiO_2 -modified membrane (spectrum b) reveal 4 and 6 unique phosphopeptides, respectively. The spin-tip enrichment provides a higher recovery of multi-phosphopeptides (e.g. m/z 3122), while the membrane enrichment favors mono-phosphopeptides (e.g. m/z 1952 and 2511). Overall, the two methods have similar recovery and binding capacity, however membrane enrichment takes about 25 min, whereas the spin-tip enrichment takes more than 1 h because of the slow passage of solution through the tip. Larger spin-tubes (3 mL) with greater binding capacity (50 μg of phosphopeptide compared to 3.5 μg for the 200 μL tip) can process more volume at one time but require more starting material and larger elution volumes. Due to the small pore radii in the membranes, mass transfer to the binding sites is sufficient to capture essentially all phosphopeptides at solution linear velocities of 5 cm/min. The Titansphere™ Phos-TiO manufacturer protocol also requires more stringent loading conditions, 0.3 vol% TFA, 60 vol% ACN and 25 vol% lactic acid, compared to the 0.1–2% TFA loading solution for the

membranes. ESI-MS will generally require removal of the lactic acid, while membrane eluates should require no further desalting.

When we attempted to enrich phosphopeptides using the Titansphere™ Phos-TiO spin tip with a 0.1% TFA loading solution, the eluate gave dominant signals of nonphosphopeptides in MALDI mass spectra (Figure S9). Under the same conditions, membrane enrichment leads to dominant phosphopeptide signals. Furthermore, we diluted the 5 fmol/μL digests to 1 fmol/μL in loading buffer I and enriched phosphopeptides from 5 mL of the diluted digests using a TiO₂-modified membrane. Spectrum 5c (1 μL of the eluate from enrichment of the diluted digests) reveals the same phosphopeptides with similar S/N ratios and intensities as enrichment of the same amount of digest in a more concentrated solution (spectrum 5b). Phosphopeptide enrichment at the 1 fmol/μL level suggests that we can further decrease the protein consumption to fmol levels if we use nanoHPLC-MS and employ the entire eluate. However, we should note that signals for phosphopeptides enriched from 5 fmol/μL solutions decrease when digested BSA (100-fold excess) is added to loading solutions.

In one experiment, we limited the loading solution volume to 20 μL. Dong and coworkers previously enriched phosphopeptides from 20-μL volumes using a monolithic capillary system.⁴⁹ Figure S10 shows that selectivity and S/N decreased compared to that when treating the same amount of peptide in 100 μL of loading buffer (Figure S8b). With the low-volume enrichment, we observed three non-phosphopeptides, whose signal intensities range from 18% to 48% of the highest phosphopeptide signal. More effective enrichment of phosphopeptides from small volumes (<20 μL) will require further optimization of the membrane system.

Enrichment of p-tau digests

The above studies suggest that TiO₂-modified membranes are attractive for enriching phosphopeptides from modestly complex samples such as digests of complexes containing fusion proteins. To test this system with a phosphoprotein with more direct relevance to biology, we enriched phosphopeptides from a digest of hyperphosphorylated tau (p-tau). Abundant in neurons, tau is a very soluble microtubule-associated protein that along with tubulin assembles and stabilizes the microtubule cytoskeleton.⁵⁰ In many neurodegenerative conditions such as Alzheimer's disease, hyperphosphorylated tau dissociates from the microtubule and aggregates into paired helical filaments and neurofibrillary tangles,³³ whose abundance correlates well with the severity of neurodegeneration of Alzheimer's disease patients. Thus, compounds that block or revert tau aggregation may have therapeutic values in treating Alzheimer's disease. Development of such potential drugs will benefit greatly from a better understanding of the site of p-tau phosphorylation. One of the candidate kinases for tau hyperphosphorylation in Alzheimer's disease is glycogen synthase kinase (GSK) 3β. In vitro, GSK-3β catalyzes tau phosphorylation that facilitates the formation of tangle-like aggregates.^{34,51–53} To prepare the p-tau subject for our TiO₂ enrichment, we employ a protein interaction module-assisted catalysis technique (PIMAX) in which GSK-3β and tau are expressed as His₆-FosB and JunD fusion proteins, respectively (Figure S11; detailed information of PIMAX to be published elsewhere). The formation of a stable FosB-JunD leucine zipper complex in *E. coli* leads to high-efficiency phosphorylation of tau. Subsequently, affinity-trapping of the His-tag with a Ni-nitrilotriacetic acid column enables isolation of the complex, which in this study was further purified through size-exclusion fast protein liquid chromatography (FPLC). Selected FPLC fractions containing GSK-3β, p-tau and a small number of bacterial proteins were pooled for MS.

Figure 6a shows the MALDI mass spectrum of a digested FPLC fraction that contained p-tau. The peptide signals stem from GSK-3β, p-tau, and unknown proteins that co-purify with

the complex. The sample also contains 0.12 M urea and 10 mM NaCl, which decrease the S/N, so the highest S/N is 20 for a peptide from GSK-3 β . Overall, the spectrum reveals 63 peptides whose signals are above 10 % of the highest signal, but none of these are phosphopeptides. Although comprehensive phosphopeptide identification in a simple system sometimes can occur without peptide enrichment,⁵⁴ this is not the case for these protein mixtures. Using the TiO₂-modified membranes with loading buffer II, we captured the phosphopeptides from 200 μ L of the same digested FPLC fraction and then recovered these peptides in 50 μ L of elution buffer. The mass spectrum of 1 μ L of the eluate spotted on a MALDI plate (Figure 6b) shows a much simpler pattern than in Figure 6a, and the S/N is as high as 100 for a phosphopeptide from p-tau. The TiO₂-modified nylon membrane isolates, concentrates (4-fold) and desalts the phosphopeptides to enable their detection. We identified seven phosphorylation sites from three phosphopeptides, P₁, P₃, and P₄ (P₂ results from in-source loss of 98 Da from P₃). Even the strongest phosphopeptide signal, P₃ at 2455.00 m/z, is not present in the mass spectrum of the membrane-loading solution, presumably because of suppression. The enriched mixture also shows signals from four unphosphorylated peptides (labeled as n in spectrum b). These signals are also present in Figure 6a, but they do not stem from either p-tau or GSK-3 β . A control experiment with tau digests without GSK-3 β stimulation (Figure S12) showed no detectable phosphopeptides after enrichment.

To identify the phosphorylation sites on p-tau, we employed ESI-Orbitrap-MS coupled with CID-, HCD-, or ETD-MS/MS and/or MSⁿ fragmentation. In CID-MSⁿ (Figure S13) the dominant sequential loss of phosphate under consecutive CID fragmentations confirms that ³⁸⁶TDHGAEIVYKS_pPVVS_pGDTS_pPR has three phosphate groups. The CID MS/MS spectrum gives 87 fragment ions resulting from breaking of 17 out of 20 peptide bonds. The HCD MS/MS spectrum shows 93 fragment ions from the breakage of 18 out of 20 peptide bonds (Figure S14). The higher energy causes more internal fragmentation, leading to additional small fragments at a low mass range compared to CID analysis. Both techniques provide high confidence for peptide identification. However, the potential for competing losses of 98 Da (either directly as H₃PO₄ from a phosphorylated Serine or Threonine residue, or as the combined losses of 80 Da (HPO₃) from a phosphorylated Serine or Threonine residue and 18 Da (H₂O) from a non-phosphorylated Serine or Threonine residue) from either the precursor ion or from the b- and y-type product ions may result in ambiguity in assigning the sites of phosphorylation within the peptide sequence.⁵⁵ In contrast, retention of phosphate groups during ETD^{56,57} allows confident assignment of phosphorylation sites (Figure 7). Two other phosphopeptides were identified using HCD analysis and the phosphate groups were assigned as ¹⁹⁵S_pGYS_pSPGSPGTPGSR and ⁴⁰⁷HLS_pNVSSTGSIDMVDS_pPQLATLADESASLAK (see mass spectra in Figure S15). Overall, we identified 7 phosphorylation sites (S195, S198, S396, S400, S404 S409 and S422) using MS analysis following enrichment with the TiO₂-modified nylon membrane.

Multiple approaches such as MS, western blotting, and Edman degradation previously revealed more than 40 phosphorylation sites in different tau isoforms,⁵⁸ making it particularly challenging to map all phosphorylation sites of a given p-tau species. However, as Figure 6b shows, the signal from the ³⁸⁶TDHGAEIVYKS_pPVVS_pGDTS_pPR (S396, S400 and S404) peptide dominates the spectrum of the enriched peptides. There are three possible reasons for this dominance. First, the peptide has a particularly high ionization efficiency. Given the presence of 3 phosphates, this seems unlikely for analysis in the positive ion mode. Second, the membrane selectively enriches the tri-phosphorylated peptide. However, TiO₂ often shows a bias toward mono-phosphorylated peptides (see Figure 5 for example).^{59,60} Third, and the most likely of the three reasons, the stoichiometry of ³⁸⁶TDHGAEIVYKS_pPVVS_pGDTS_pPR phosphorylation may surpass all other tryptic

phosphopeptides of p-tau. Using traditional methods of preparing p-tau with a commercial GSK-3 β , the Gamblin group phosphorylated tau with 3 moles of phosphate incorporated per mole of tau. Similarly, the Reynolds group reported 2–4 moles phosphate/mole of Tau.^{52,61} They also found a S396-S400-S404 tri-phosphorylated peptide. We thus suspect that these three residues are the most preferred targets for GSK-3 β . This phenomenon is also consistent with the observation that GSK-3 β activity can be enhanced by pre-existing, priming phosphorylation that lies 4 residues C-terminal to the next phosphorylation site.⁶² On the other hand, whether any of these three phosphorylation sites plays a key role in Alzheimer's disease remains to be assessed. Further refinement of the phosphorylation, sample preparation, and enrichment procedures will likely yield more comprehensive mapping of GSK-3 β -phosphorylated residues in p-tau. Identification of the most dominant and other substoichiometric phosphorylation sites may prove useful in designing drugs to prevent the pathological hyperphosphorylation of tau and even the appearance of the neurofibrillary tangles.⁵⁹ Glycosylation and other post-translational modifications could accompany phosphorylation on tau,⁶³ but such modifications in our *in vivo* GSK3-tau system are unknown so far and not the focus of this research.

CONCLUSIONS

Layer-by-layer modification of porous substrates with PSS/TiO₂ nanoparticle films is a convenient method for rapidly producing membranes that enrich phosphopeptides. The TiO₂-modified membranes have a binding capacity of 540 nmol phosphoangiotensin/membrane for a 22-mm diameter disc, and a capacity of 3 nmol in membranes designed to handle flexible volume (10's of μ L to 5 mL) samples. Because of low backpressure and minimal diffusion limitations, modified membranes can rapidly enrich phosphopeptides from 5-mL samples with low concentrations (1 fmol/ μ L). Moreover, the nanoparticle-based membrane enrichment does not require the addition of excluders (e.g. lactic acid or DHB) to loading solutions. Under sufficiently acidic conditions (2% TFA), the membranes selectively enrich phosphopeptides from a digest containing a 1:100 molar ratio of phosphoprotein to non-phosphoprotein. Thus, the membranes may prove useful in enriching phosphopeptides from modestly complex mixtures such as fusion proteins or immunoprecipitates. We used these membranes to enrich phosphopeptides from p-Tau digests, and MS analysis of the enriched eluate revealed 7 phosphorylation sites, three of which seem dominant. Identification of such sites might aid the design of therapeutic drugs that inhibit phosphorylation.^{64,65}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Cartoon of a membrane containing TiO_2 for phosphopeptide enrichment and a miniaturized membrane holder with a syringe pump.

Figure 2. Cross-sectional SEM images of nylon membranes before (a) and after (c) coating with a PSS/TiO₂ nanoparticle film. Images b and d show the EDX Ti maps for the images in (a) and (c), respectively. In (b) and (d) Ti signals are proportional to the intensity of the red color, and image b indicates the noise of the measurement.

Figure 3.

Breakthrough curve for the passage of 20 μM phosphoangiotensin (in loading buffer I, 0.1% TFA) through a 22 mm-diameter nylon membrane modified with a $(\text{PSS}/\text{TiO}_2)_2$ film. The flow rate through the membrane was 2 mL/min. Angiotensin served as an internal standard, and the large error bars (standard deviation of 40%), result from the uncertainty inherent in quantitative analysis based on MALDI-MS.

Figure 4.

(a) MALDI mass spectrum of 1 μL of a loading solution containing 30 fmol (each) of digested α -casein, β -casein and ovalbumin and 3 pmol of BSA in 260 mM urea. (b) Mass spectrum of the phosphopeptides enriched from 500 μL of the above loading solution by adsorption on a TiO_2 -modified nylon membrane, rinsing with 3 mL washing buffer II, and recovery with 10 μL of elution buffer. One μL of the eluate was spotted on the MALDI plate prior to addition of matrix.

Figure 5. MALDI mass spectra of 1- μ L eluates from (a) a Titansphere™ Spin-Tip loaded with 1 mL of 5 fmol/ μ L digested proteins, (b) a TiO₂-modified membrane loaded with 1 mL of 5 fmol/ μ L digested proteins and (c) a TiO₂-modified membrane loaded with 5 mL of 1 fmol/ μ L digested proteins. Assuming full recovery, each 1- μ L eluate contains 500 fmol of phosphopeptides from digested α -casein, β -casein and ovalbumin. Total eluate volumes were 10 μ L.

Figure 6.

(a) MALDI mass spectrum of a digested FPLC fraction that contained approximately 800 fmol of GSK-3 β -p-tau complex along with associated proteins; (b) The corresponding mass spectrum of peptides enriched from the above solution by adsorption on a TiO₂-modified nylon membrane and subsequent elution. The figure shows the sequences of 4 phosphopeptides, one of which, P_2 , results from in-source P_3 dephosphorylation. Four major unphosphorylated peptides are labeled with n.

Figure 7.

ESI-Orbitrap ETD-MS/MS spectrum of the $[M+3H]^{3+}$ precursor ion of $^{386}\text{TDHGAEIVYKS}_p\text{PVVS}_p\text{GDTSpPR}$ (m/z 819.34): The sequence shows cleavage sites for formation of c and z ions.